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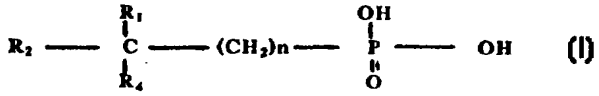

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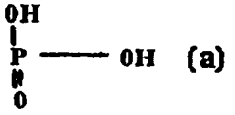

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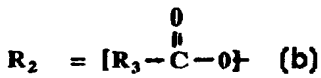

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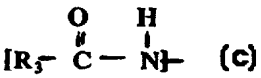

(c)

(57) Abstract

Disclosed are organo phosphonic acids having formula (I) where: n = 0 or 1; $R_1$ = H or (a); $R_2$ = (b) or (c); $R_3$ = an alkyl group containing > 10 carbon atoms; and $R_4$ = H or $CH_3$, that provide improved bio-availability of drugs and nutrients by permiabilising cell membranes. The products are prepared by the method of reacting an acid chloride of long chain aliphatic acid having 10-20 carbon atoms with an alkyl phosphonic acid derivative containing an active amino or hydroxy group in chloroform at temperatures below 5 °C. Especially useful are the reaction products of palmitoyl or lauroyl chloride with ethane-1-hydroxy-1,1-diphosphonic acid, 2-amino-ethyl phosphonic acid or 1-amino-ethyl phosphonic acid.

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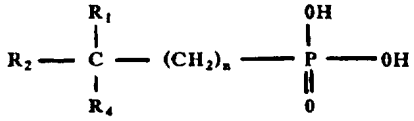
# ORGANO PHOSPHOROUS COMPOUNDS

The present invention relates to organo phosphorous compounds that provide improved bio-availability of drugs and nutrients by permiabilising cell membranes. In particular the compounds may be used as animal feed additives to increase nutrient uptake, pigment absorption in aqua-cultures and for the nanoencapsulation of drugs for drug delivery in vivo, in a way similar to lysophospholipids and phospholipids. A suggested mechanism for the improvement in bio-availability is given in the paper by David Garnett and Robin Jones in The Genetic Engineer and Biotechnologist, Vol.13, No.2, 1993 and in International Application W094/22324.

Compounds of the type referred to above are available as purified natural compounds and as a result are substantially impure. There is, therefore, a need for compounds of higher purity, such as may be obtained by chemical synthesis, which will have improved thermal stability and resistance to enzymatic attack, when compared to phospholipids.

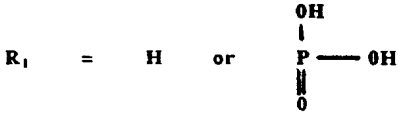
The organo-phosphorous compounds of the present invention are phosphonolipids preferably prepared by the reaction of a long chain alkyl acid chloride with an organophosphonic acid derivative preferably including a reactive hydroxy or amino residue.

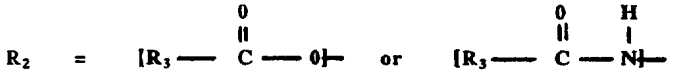
From one aspect the present invention provides an organo phosphonic acid compound having the formula:-



where:-

n = 0 or 1





$R_3$ = an alkyl group containing > 10 carbon atoms

$R_4$ = H or $CH_3$

Preferably $R_4$ is a methyl group.

The alkyl acid chlorides have a chain length of 10 or more carbon atoms and preferably not more than about 20 carbon atoms; particularly suitable are the acid chlorides of palmitic and lauric acids.

Particularly suitable phosphonic acid derivatives are ethane-1-hydroxy-1, 1-diphosphonic acid (Etidronic acid) and 1 and 2 amino-ethyl phosphonic acids.

From another aspect, therefore, the present invention provides a method for preparing the organo phosphorous compounds of the present

invention suitable for use as animal feed additives, pigment absorption in aqua-culture and nanoencapsulation drugs for drug delivery in vivo, comprising reacting an acid chloride of a long chain aliphatic acid with an alkyl phosphonic acid derivative containing an active amino or hydroxy group in a non-aqueous medium, preferably chloroform, at low temperatures, washing with cold water and subsequently drying.

Preferably the reaction temperature is kept below 5°C and most preferably in the range 3-5°C.

Preferably also the reactants are employed in stoichiometric proportions.

The invention also provides an organophosphonic acid as defined above for use as an animal feed additive, to improve pigment absorption in aqua culture or for the nanoencapsulation of drugs for drug delivery in vivo.

From another aspect the invention provides an organo phosphorous compound of the present invention capable of being used as an animal feed additive prepared by the reaction of ethane-1-hydroxy-1, 1-diphosphonic acid and palmitoyl chloride in a non-aqueous medium at temperatures below 5°C.

From a further aspect the invention provides a method for the manufacture of an organo-phosphonic acid derivative of the present invention suitable for use in the nano-encapsulation of drugs for drug delivery in vivo by the reaction of 1 or 2-amino ethyl phosphonic acid and palmitoyl chloride in a non-aqueous medium at temperatures below 5°C.

The following examples are illustrative but non-limitative of the present invention.

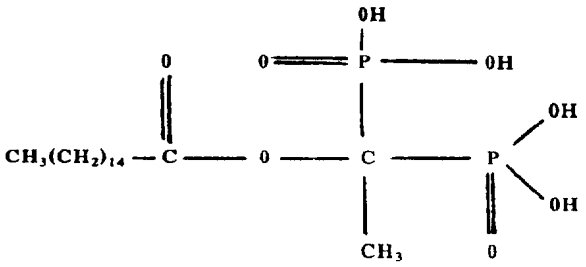
Example 1

Preparation of Ethane-1-Palmitoyl-1,1 Diphosphonic Acid

A glass 5-litre 3 necked reacton flask equipped with a stirrer is charged through one neck with 206 grams of Etidonic acid (ethane-1-hydroxy-1, 1-diphosphonic acid) dissolved in 1 litre of chloroform and the solution cooled to a temperature in the range 3 to 5°C. 275 grams of palmitoyl chloride are added slowly through one neck with vigorous stirring whilst maintaining the temperature at 3-5°C. Hydrochloric acid evolved in the reaction is withdrawn through the other neck and passed through a sodium hydroxide scrubber. Stirring is continued for 60 minutes after the addition of the palmitoyl chloride is complete. At the end of the reaction the contents of the reaction flask are poured into cold water and the chloriform and unreacted palmitoyl chloride, in the form of palmitic acid (a wax) separated off. The resultant product is further washed with cold water to remove any trace of unreacted etidonic acid. After repeated washing the product is filtered off and dried under vacuum.

The product yield was 90% of theoretical.

The product is soluble in water at about 70°C to provide a solution of pH 10.5 and is characterised by a Near Infra Red (NIR) spectra (measured on an NIR Systems Seris 5000 spectrometer), shown inFigure 1. It is believed that the product has the structure:-



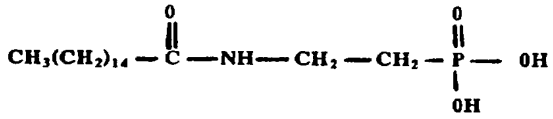
The effect of Ethane-1-Palmitoyl-1,1-Diphosphonic Acid on pigment uptake in fish is shown in example 9 below.

## Example 2

The process of Example 1 was repeated except that the etidonic acid was replaced by 125 grams of 2-amino-ethyl phosphonic acid.

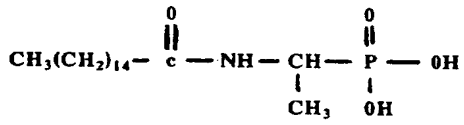
The product yield was 90% of theoretical. The product dissolved in water at 60°C to provide a solution having a pH of 3.33.

The product had a NIR spectra as shown in Figure 2 and is believed to have the structure:



## Example 3

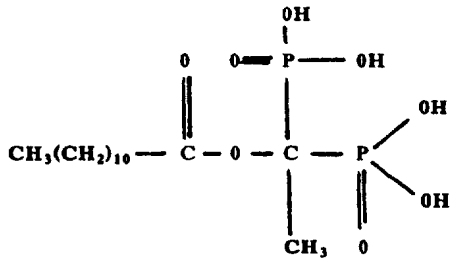
Example 2 was repeated using 1 amino ethyl phosphonic acid in place of 2 amino ethyl phosphonic acid. The product yield was 60% and had an NIR spectra as shown in Figure 3. The product was soluble in water at 60°C to give a solution of pH 3.76 and is believed to have the structure shown below:



Example 4

Example 1 was repeated except that the palmitoyl chloride was replaced by 219 grams of lauroyl chloride.

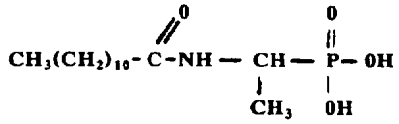
The product yield was 23.2% of theoretical and the product dissolved in water at 60°C to give a solution having a pH of 10.42. The product is identified by is NIR spectra shown in Figure 4 and is believed to have the structure shown below:-



Example 5

Example 3 was repeated replacing palmitoyl chloride with 219 gram of lauroyl chloride. The product yield was 20% of theoretical.

The product is believed to have a structure as shown below:-



The use of octanoyl chloride in place of palmitoyl chloride with either etidronic acid or 2 amino ethyl phosphonic acid did not produce any useful product.

The in vitro effect of the product of Example 1 on Inulin uptake in BHK cells is shown in Example 7 below.

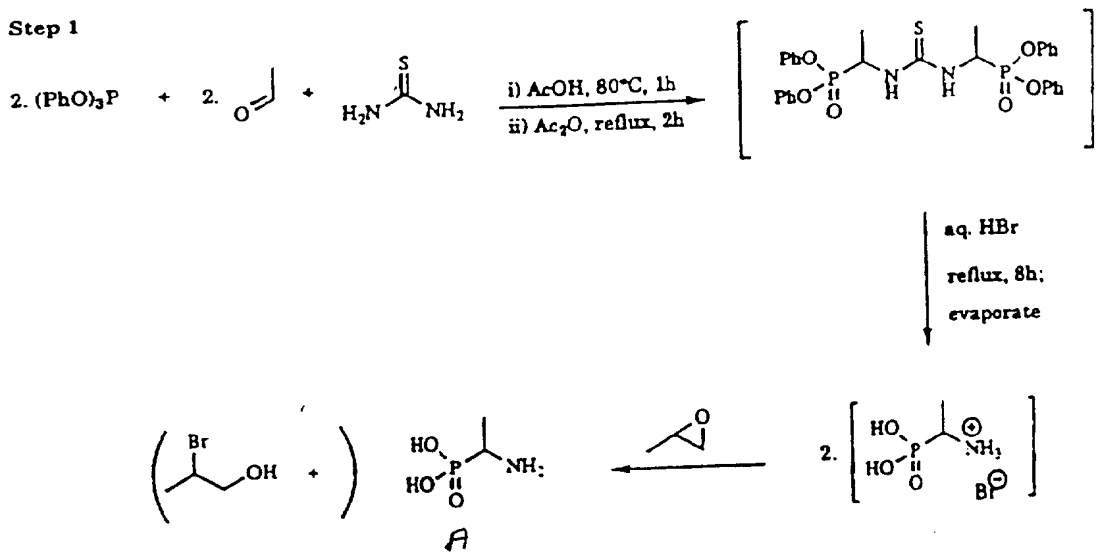
Example 6

**Preparation of 1 -Palmitoylaminoethylphosphonic Acid**

The preparation was conduced in three steps.

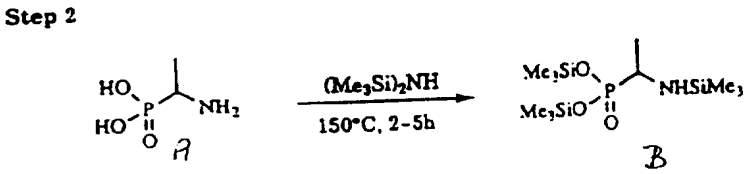
**Step 1**

Thiourea (9.89g.130 mmol) was added to a solution of acetaldhyde(18.57 ml.325 mmol)and triphenylphosphite (68.13 ml,325 mmol) in acetic acid (130 ml) and the resulting solution stirred and heated at 80°C for 1hr to give an orange solution. Acetic anhydride (325 ml) was then added and the resulting solution refluxed for 2h to give a dark brown solution. 37% Aqueous hydrogen bromide (325 ml) was then added very carefully dropwise, via the condenser, and the resulting solution refluxed for 8h then cooled and rotary evaporated. The dark residue was dissolved in ethanol (260 ml) and methyloxirane added slowly until pH6 was reached. This addition resulted in the immediate precipitation of the aminophosphonic acid A shown as the product of the reactions outlined below. The solid was allowed to settle and the liquid supernatant decanted. Fresh ethanol (250 ml) was added and the mixture briefly refluxed, then cooled and the ethanol decanted. This washing was repeated until most of the brown coloration was removed. The solid was finally dried under high vacuum to give the aminophosphonic acid A (8.23g,50.6%) as a slightly coloured solid, m.p.<290°C(dec.).




**Step 2**

A mixture of the aminophosphonic acid A (8.23g.65.8 mmol) and hexamethyldisilazane (55.5ml,263 mmol) was heated on an oil bath at 150-160°C until all the solid dissolved (2-5h). Excess hexamethyldisilazane was evaporated and the residue was distilled to give the N,0,0-trisily derivative B (17.50g,86%), as a colourless oil, b.p.140°C at 15mm Hg, the structure of which is outlined in the reaction illustrated below.

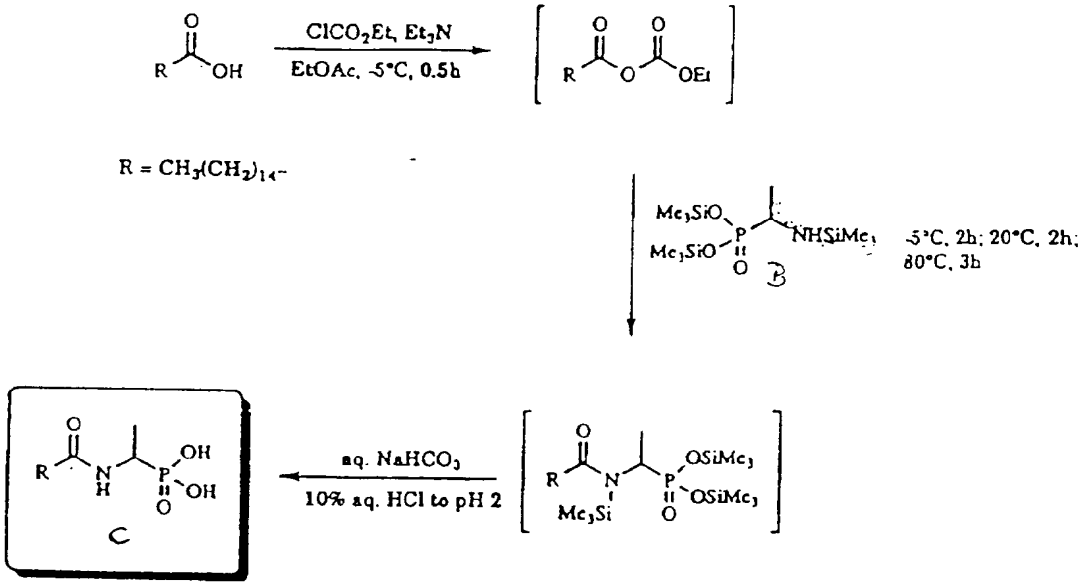



**Step 3**

A solution of palmitic acid (13.18g,51.4 mmol) in dry ethyl acetate (80ml) was stirred at -5°C then treated sequentially with triethylamine (17.15ml,5.4mmol) and ethyl chloroformate (5.57g,51.4mmol), the latter added dropwise. The resulting cloudy mixture was stirred at -5°C for 0.5h, then a solution of tris-silyl derivative B in ethyl acetate (35ml) was

added dropwise and the resulting mixture stirred at the same temperature for 2h, then for 2h without cooling and finally for 3h at 80°C. The volatiles were removed from the cooled mixture by rotary evaporation. The residue was dissolved in saturated aqueous sodium bicarbonate and the resulting solution acidified to pH 2 using 10% hydrochloric acid and the precipitated acylamino-acid C filtered off. The crude solid was washed with methanol to remove residual palmitic acid then dried under high vacuum to leave the 1-Palmitoylaminoethylphosphonic acid C (16.0g,86%) as a white solid.

Step 3




The NMR spectra for the resultant lysophosphonolipid is shown in Fig.5.

Example 7

BHK 21(clone13)cells were cultured in the normal way using G-MEM (Sigma G5154) supplemented with 10% FBS (Sigma F2442), 10ml/L of 200mM L-Glutamine (Sigma G7513) 5% TSB (Sigma T8159) and 10 ml/L antibiotic/antimycotic solution (Sigma A9909). The cells were cultured at 37°C with 5%$C0_2$. The cell line was sourced from the European Collection of Animal Cell Cultures at the Centre for Applied Microbiology and Research at Porton Down.

These cells were cultured in 24 well plates (Sigma M9655). All experiments were performed in triplicate.

Cells in the wells were exposed to 1μCi of C-$^{14}$ Inulin (Sigma 30,480-8) prepared in Hanks Balanced Salt Solution (Sigma H9394) from a 75μCi stock solution. These were co-exposed to varying concentrations of the product of Example 1. The cultures were left for 1 hr and then the media was removed by aspiration. The adhered cells were then washed twice in Hanks' solution and then the cells were trypsinised using Trypsin-EDTA solution (Sigma T5775) and centrifuged to a pellet at 1000rpm in pre-weighed Eppendorf tubes. The cell pellet was then lysed using distilled water. The Eppendorfs are then re-spun and aliquots of the D-water (100μ) were then removed and mixed with SigmaFluor Scintillation cocktail (Sigma S4398) and read in a scintillation counter. Results are converted into DPM per mg/cell weight after the Eppendorfs are re-weighed.

Results are plotted on the graph shown in Figure 6 and clearly show a bi-phasic response, where at very low concentrations the flux of Inulin into the cells is reduced and at higher doses the influx is dramatically increased whilst Inulin is a large compound and is not particularly typical of compounds which one might wish to preferentailly absorb. The same effect can be observed with other sized molecules.

Inferences about the emulsion forming properties of phosphonolipids and phosphonolipid mixtures can be made from the determination of the emulsifying power of Lecithin formulations. An Empirical method that determines the percentage of oil in water emulsion that is stable for an excess of 24 hours is as follows:-

Measure 100 ml amounts of distilled water and vegetable oil into a beaker. Add the test sample of lecithins (normally 1 gram). Using the high speed rotor action blender homogenise the mixture for 1 minute at 25°C. Immediately empty the container into a measuring flask and leave at 25°C for 24 hours. At the end of 24 hours calculate the percentage oil-water emulsion remaining.

An assay carried out comparing lecithin with a 90% lecithin - 10% of example 1 product gave the following results:-

Contol 0.0010
Lecithin 0.1948
90% Lecithin - 10% product Example 1 - 0.222

These results show a 12.3% improvement in the emulsifying power.

The improvement in stability of Miscelles formed the inclusion of the product of Example 2 is indicated in Example 8 below.

## Example 8

Liposomes were created as follows:-

An aqueous 1% $^{v}/_{v}$ dispersion of the product of Example 2 was prepared by homogenisation using an Utra-Turrex T25 homogeniser operating at 14,000 rpm for approximately 30 seconds. Two sets of

liposomes were created, the first test sample was formed using pure phosphatidylcholines and the second set made using 10% of the product of Example 2 in the formulation. Liposome concentration was determined spectro-photometrically using a Cecil Series 2 Machine at 500 NM wavelength. The liposome preparations were examined microscopically to ensure calibration of the spectrophotometer. The miscelle preparations were subject to a 30°C heat regime for periods of 10 minutes and 3 hours and 60°C for 30 minutes using a heated water bath. The results of the analysis are given below.

| | 10 minutes | 3 hours | 60°C-30 mins |
|---|---|---|---|
| Lecithin | | | |
| Lecithin + 10% | 1.31 | 1.288 | 0.975 |
| Product of Example 2 | 1.34 | 1.338 | 1.062 |
| Net Increase | 0.022% | 3.88% | 8.92% |

The results indicate that there is a significant improvement in the stability of miscelles at elevated temperatures in the presence of phospholipids formed according to the present invention.

## Example 9

Effect of Ethane-1-Palmitoyl-1,1-Diphosphonic Acid (Corbinol) on pigment uptake in salmon.

350 post smolt Atlantic salmon originating from a pure Mowi mixed sex stock, aged 24 months from hatch and being an initial average weight of 1.2kg were used in this trial.

The details of the five holding facilities used are given below:

A-Four adjacent 5m trial tanks
B-One 12m production tank (sea site control)

| | A | B |
|---|---|---|
| Diameter | 5.0m | 12.0m |
| Wall depth | 1.2m | 1.8m |
| Water depth | 0.9m | 1.6m |
| Cubic capacity | 17.6m | 180.0m |

Construction: Concrete tanks set into ground with flat base and smooth internal finish

Inlet: A Single 100mm inlet on tank surface directing incoming water round edge of tank. Water flow controlled with butterfly valve - maximum capacity 2000 litres per minute

B Single 200mm inlet on tank surface directing incoming water round edge of tank. Water flow controlled with slide valve - maximum capacity 2000 litres per minute.

Outlet: A 0.6m x 0.6m diameter flat scree in middle of tank base leading into 150mm discharge pipe.

B 0.5m x 0.8m diameter flat scree in middle of tank base leading to a 200mm discharge tank.

Water Supply:

A Tank inlets fed from an 11kw centrifugal pump (with standby) drawing water from main salt water header tank. Header tank fed by two separate pumping systems drawing water from a minimum depth of 3m below surface. Salinity 27ppt to 31ppt. Water screened with 15mm grid before entering pump. Water passes once through tanks (no recirculation) with a retention time of less than one hour.

B Tank inlet fed directly from main salt water header tank.

Aeration: Each tank fed by its own air diffuser supplied with air from the main farm blower. These can be used to elevate the oxygen levels in normal conditions and would keep the fish alive for several hours in the event of a water supply failure.

Level Control A External stand pipe in shared drain sump, adjacent to tank.

B External stand pipe.

Jump netting 85% shade netting 200mm high around tank circumference and predator lines.

System failure indication:

Low level float switch in each tank. Both connected to central alarm monitored 24 hrs.a day.

Automatic feeds:

Each tank fitted with a single 3kg capacity clockwork feeder suspended above the water at edge of tank.

The fish were fed to appetite three times a day by hand and supplemented with automatic feeding. About 50% of the total daily intake as calculated from standard commercial feeding tables was supplied by hand. Suitable adjustments were made to the feed rate calculation on a weekly basis to allow for the increasing weight of the fish.

Mortalities were removed immediately, their individual weights recorded and the cause of death assessed. Any disease treatments considered necessary through the course of the trial were applied to all tanks to minimise possible differences between tanks caused by the treatments.

Water flows through the tanks were maintained to ensure minimum dissolved oxygen levels of 7.0mg/litre. Oxygen levels were monitored

daily and if necessary water flows adjusted to maintain the tanks in balance. The tanks were brushed regularly to prevent the build-up of waste material and high levels of suspended solids.

Water temperature was recorded daily.

When the fish were weighed the following procedure was adopted:-

The water flow into the tank was switched off and the level dropped to 20cm. Oxygen levels were maintained with a diffuser placed inside the tank. Anaesthetic (ethylp-amino benzoate dissolved in methylated spirit) was then added to the water to sedate the fish. The fish were then netted into a 40 litre capacity bucket, containing 15 lites of water and weighed in bulk using a Salter 50kg capacity hanging spring balance. In this way stress levels and physical damage were kept to a minimum.

The diets of the fish were as follows:-

| | | |
|---|---|---|
| Diet A | - | 55ppm astaxanthin |
| Diet B | - | 55ppm astaxanthin + Corbinol (Ethane-1-Palmitoyl-1 1-Diphosphonic Acid) |
| Diet C | - | Standard trouw 75ppm Astaxanthin (Control). |

The 350 fish were split equally between the five tanks (70 fish per tank). Diet A was fed to tanks 1 and 3; Diet B was fed to tanks 2 and 4 (all type A tanks); Diet C was fed to the sea site tank (Type B tank).
The pellet size of the fish diets ws 6.5mm.

The experiment ran for 36 days.

Total weights of the fish in each tank were taken at the beginning of the trial and at the end of the trial. Samples of fish for carcass analysis were taken after 4 weeks.

Samping was carried out at the following times:-

10 fish at start of trial for baseline;

10 fish from each tank after four weeks;

All remaining fish to be sampled at end of 36 day period.

Samples were analysed for HPLC astaxanthin level immediately after culling.

After the fourth week analysis was conducted and showed greatest spread of pigmentation in group 1 and consequently more fish were selected from group one to analyse with HPLC. HPLC analysis was carried out on 25% of the fish whilst a Roche Fan score was determined for all fish.

Table 1 tabulates the statistics recorded throughout the trial.

Figure 7 is a graphical representation of the weight of the fish fed on each of the three diets at the end of the trial.

Figure 8 is a graphical representation of the Roche Fan scores of the fish fed on each of the three diets at the end of the trial.

The results shown in figures 7 and 8 were calculated using standard derivation methods and allowed for the difference in the number of fish fed the control diet compared to those fed diets A and B.

Figure 7 shows that the fish fed the nutrient, astaxanthin, together with the organo-phosphorous compound, Corbinol, exhibited a greater increase in weight than the fish fed on the diets not including the organo-phosphorous compound.

Figure 8 shows that the fish fed the nutrient, astaxanthin, together with the organo-phosphorous compound, Corbinol, exhibited a higher Roche Fan score than those fish fed diets not including the organo phosphorous compound.

TABLE 1

**Trial Statistics for example 9**

| Tank | 1 | 2 | 3 | 4 | Seasite |
|---|---|---|---|---|---|
| Diet | A | B | A | B | C |
| Start Date | 27.2.95 | | | | |
| Finish Date | 4.4.95 | | | | |
| Number of Days | 36 | 36 | 36 | 36 | 36 |
| Start number | 70 | 70 | 70 | 70 | 70 |
| Number of mortalities | 0 | 0 | 0 | 0 | 0 |
| Final number | 70 | 70 | 70 | 70 | 70 |
| Start Total weight | 80.4 | 84.7 | 82.8 | 86.5 | 68.6 |
| Finish Total weight | 97.5 | 99.5 | 96.1 | 106.2 | 86.1 |
| Mortality weight | 0 | 0 | 0 | 0 | 0 |
| Gain in weight | 17.1 | 14.8 | 13.3 | 19.7 | 17.5 |
| Start average weight | 1.149 | 1.210 | 1.183 | 1.236 | 0.980 |
| Finish average weight | 1.393 | 1.421 | 1.373 | 1.517 | 1.230 |
| % increase | 21.3 | 17.5 | 16.1 | 22.8 | 25.5 |
| Specific growth rate (%day) | 0.54 | 0.45 | 0.410 | 0.57 | 0.63 |
| Feed consumed kgs | 15.8 | 14.2 | 15.5 | 18.0 | 0 |
| Feed conversion ratio | 0.92 | 0.96 | 1.17 | 0.91 | 0 |
| Average temperature | 7.9 | | | | |

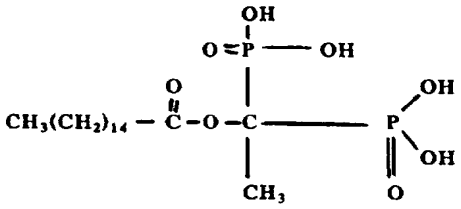
Diets: A Trouw 65, 55ppm astaxanthin

B Trouw 65, 55ppm astaxanthin + corbinol

C Control Trouw 65, 75ppm astaxanthin

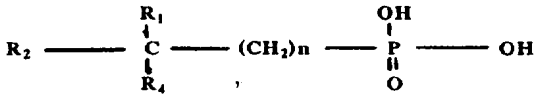
Diets supplied direct from Trouw

Corbinol (Ethane-1-Palmitoyl-1,1-Diphosphonic acid)



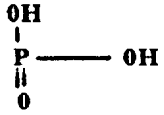
CLAIMS:

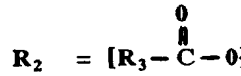
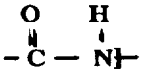
1. An organo phosphonic acid compound having the formula:



where:-

n = 0 or 1

$R_1$ = H or 

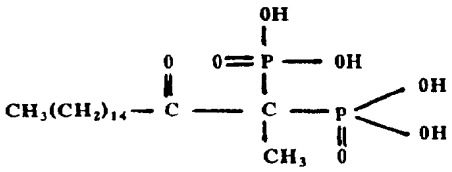
$R_2$ =  or 

$R_3$ = an alkyl group containing > 10 carbon atoms

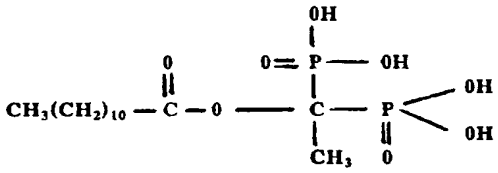
$R_4$ = H or $CH_3$

2. An organo phosphonic acid compound according to claim 1 wherein $R_4$ is a methyl group

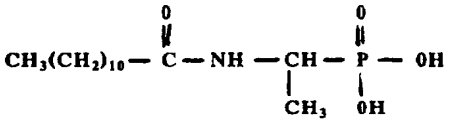
3. An organo phosphonic acid according to claim 2 having the formula:-



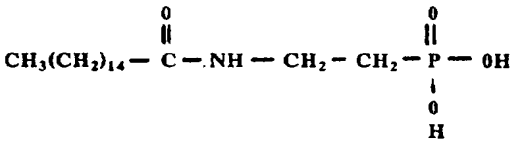
4. An organo phosphonic acid according to claim 2 having the formula:-



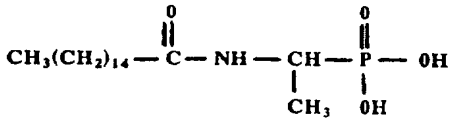
5. An organo phosphonic acid according to claim 2 having the formula:-



6. An organo phosphonic acid according to claim 1 having the formula:-



7. An organo phosphonic acid according to claim 2 having the formula:-



8. A method for preparing the organo phosphonic acid compounds of claim 1 comprising reacting an acid chloride of a long chain aliphatic acid with an alkyl phosphonic acid derivative containing an active amino or hydroxy group in a non-aqueous medium at low temperatures, washing with water and drying.

9. A method for preparing organo phosphonic acid compounds according to claim 8 wherein the non-aqueous medium is chloroform.

10. A method of preparing organo phosphonic acid compounds according to claims 8 or 9 wherein the temperature is kept below 5°C.

11. A method of preparing organo phosphonic acid compounds according to claim 10 wherein the temperature is in the range of 3-5°C.

12. A method of preparing organo phosphonic acid compounds according to any one of claims 8-11 wherein said long chain aliphatic acid chloride contains less than 20 carbon atoms in the chain.

13. A method for preparing organo phosphonic acid compounds ccording to claim 12 wherein the acid chloride is palmitoyl or lauroyl chloride.

14. A method for preparing organo phosphonic acid compounds according to any of claims 8-13 wherein the phosphonic acid derivative is ethane-1-hydroxy-1,1-diphosphonic acid (Etidonic acid) or 1 or 2 aminoethylphosphonic acid.

15. A method of preparing organo phosphonic acid compounds according to any of claims 8-14 wherein the reactants are employed in stoichiometric proportions.

16. The organo phosphonic acid compound of any one of claims 1-7, when used as an animal feed additive, to improve pigment absorption in aqua culture or for the nanoencapsulation of drugs for drug delivery in vivo.

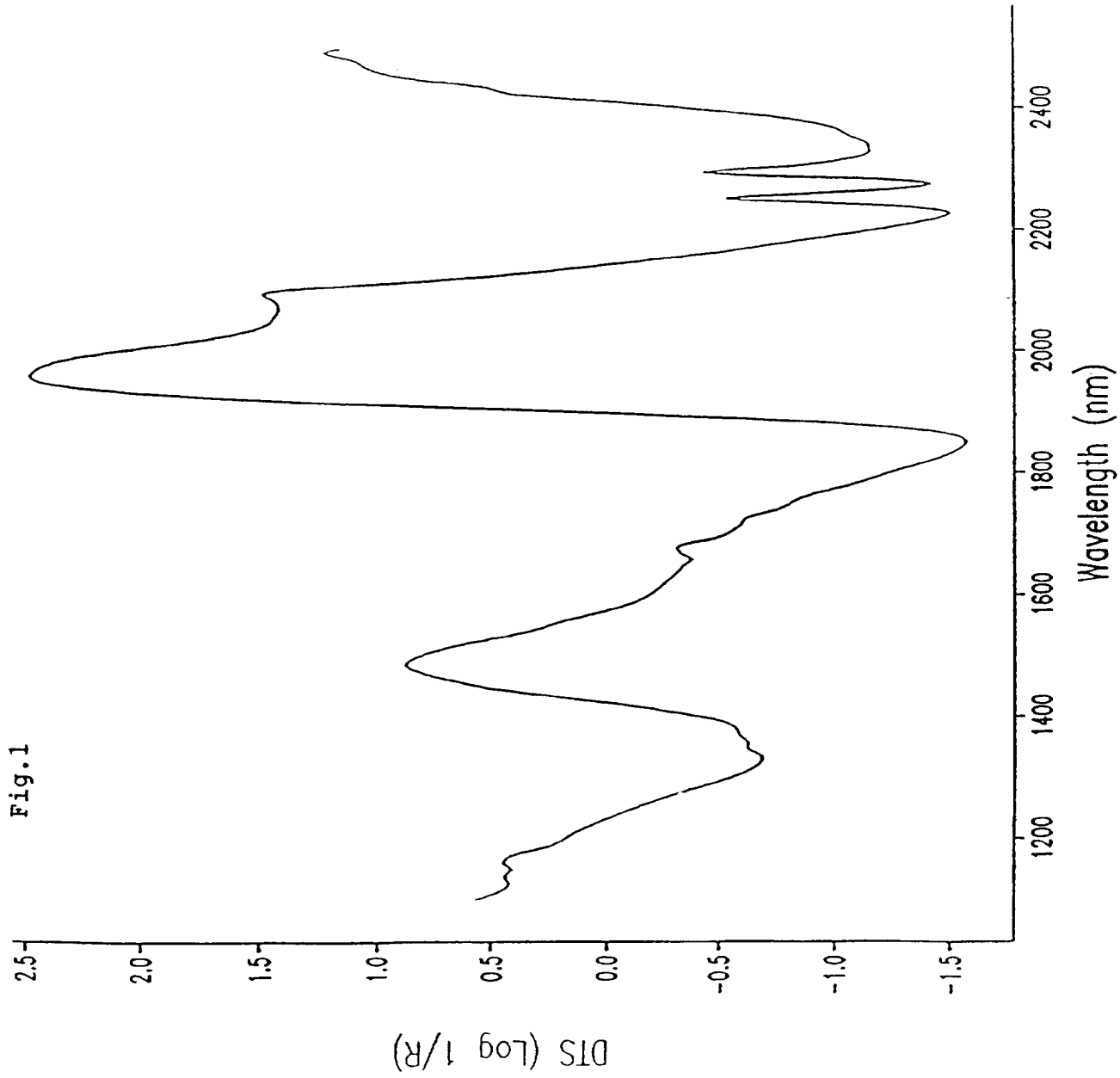

DTS (Log 1/R)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
1200
1400
1600
1800
2000
2200
2400
Wavelength (nm)

Fig.1

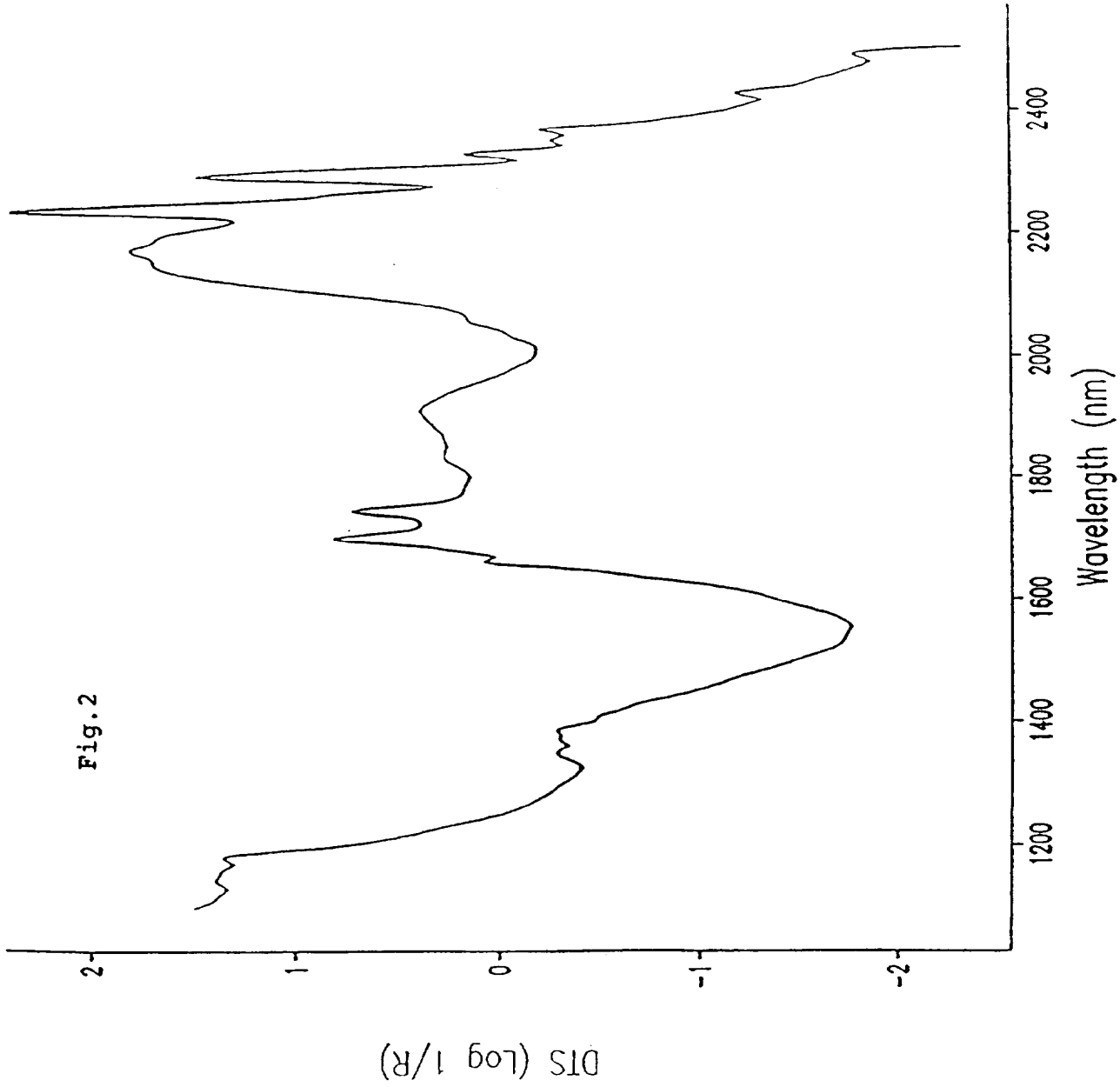

DTS (Log 1/R)
2
1
0
-1
-2
1200
1400
1600
1800
2000
2200
2400
Wavelength (nm)

Fig.2

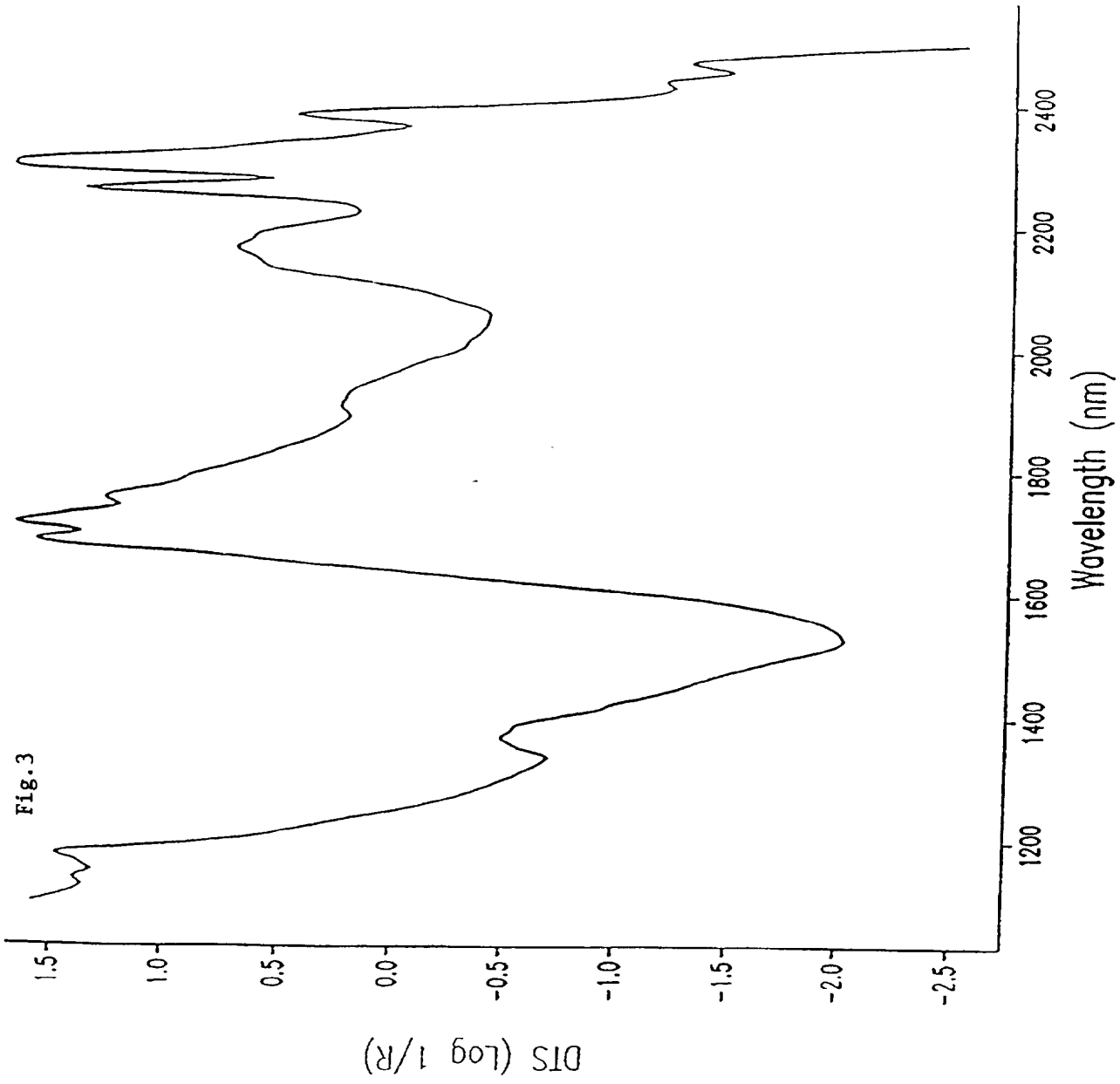

DTS (Log 1/R)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
1200
1400
1600
1800
2000
2200
2400
Wavelength (nm)

Fig.3

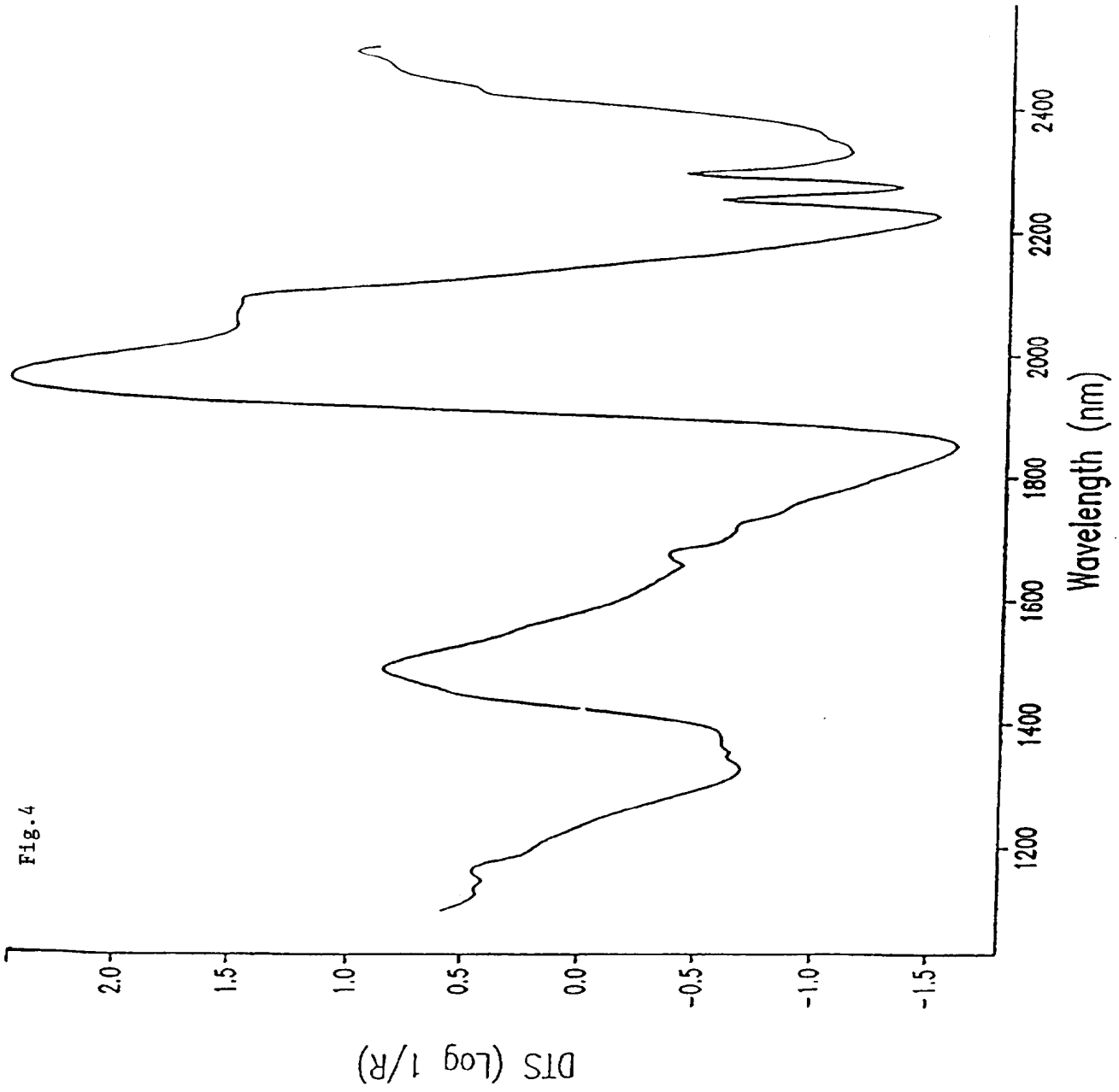

DTS (Log 1/R)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
1200
1400
1600
1800
2000
2200
2400
Wavelength (nm)

Fig.4

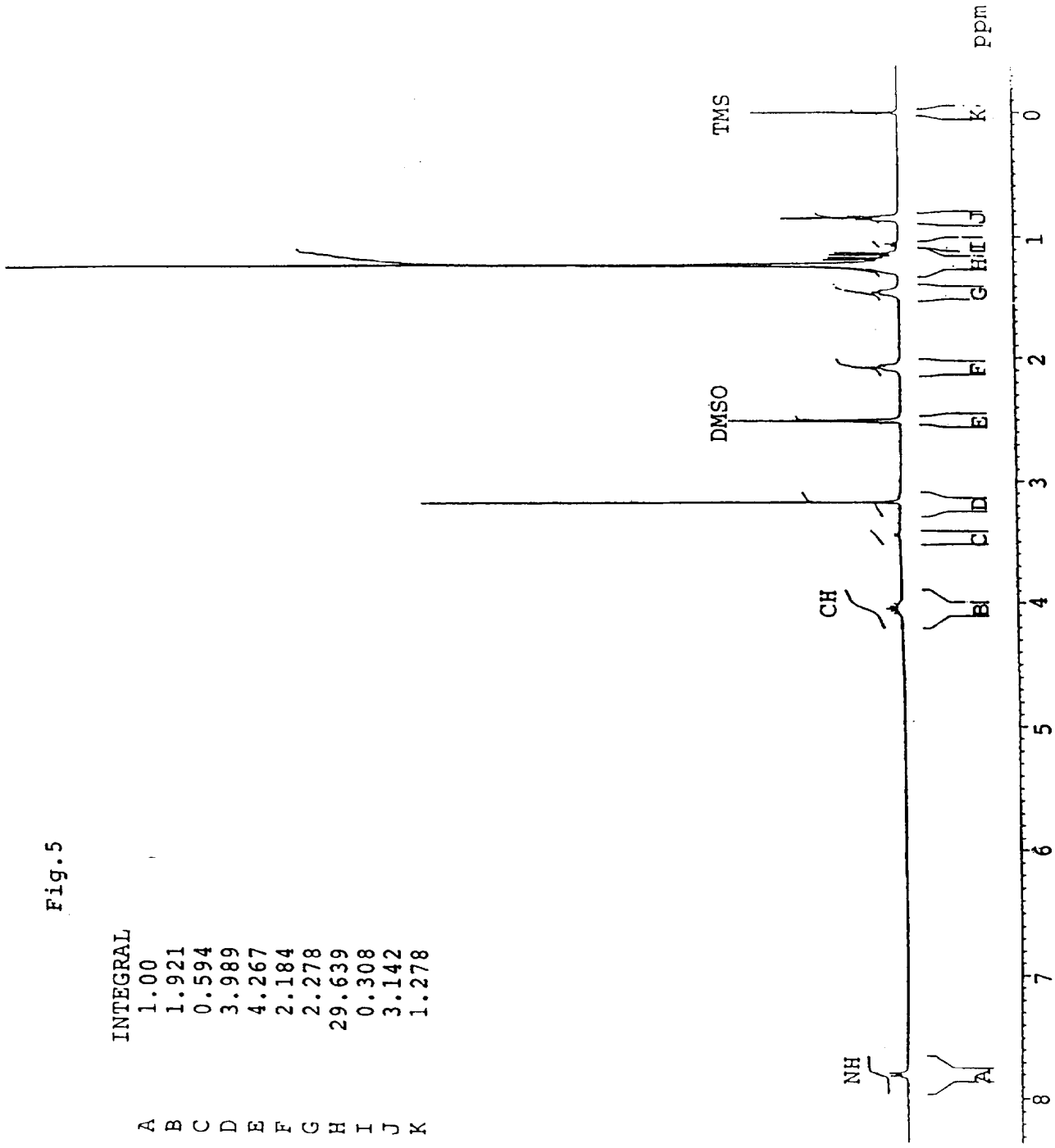
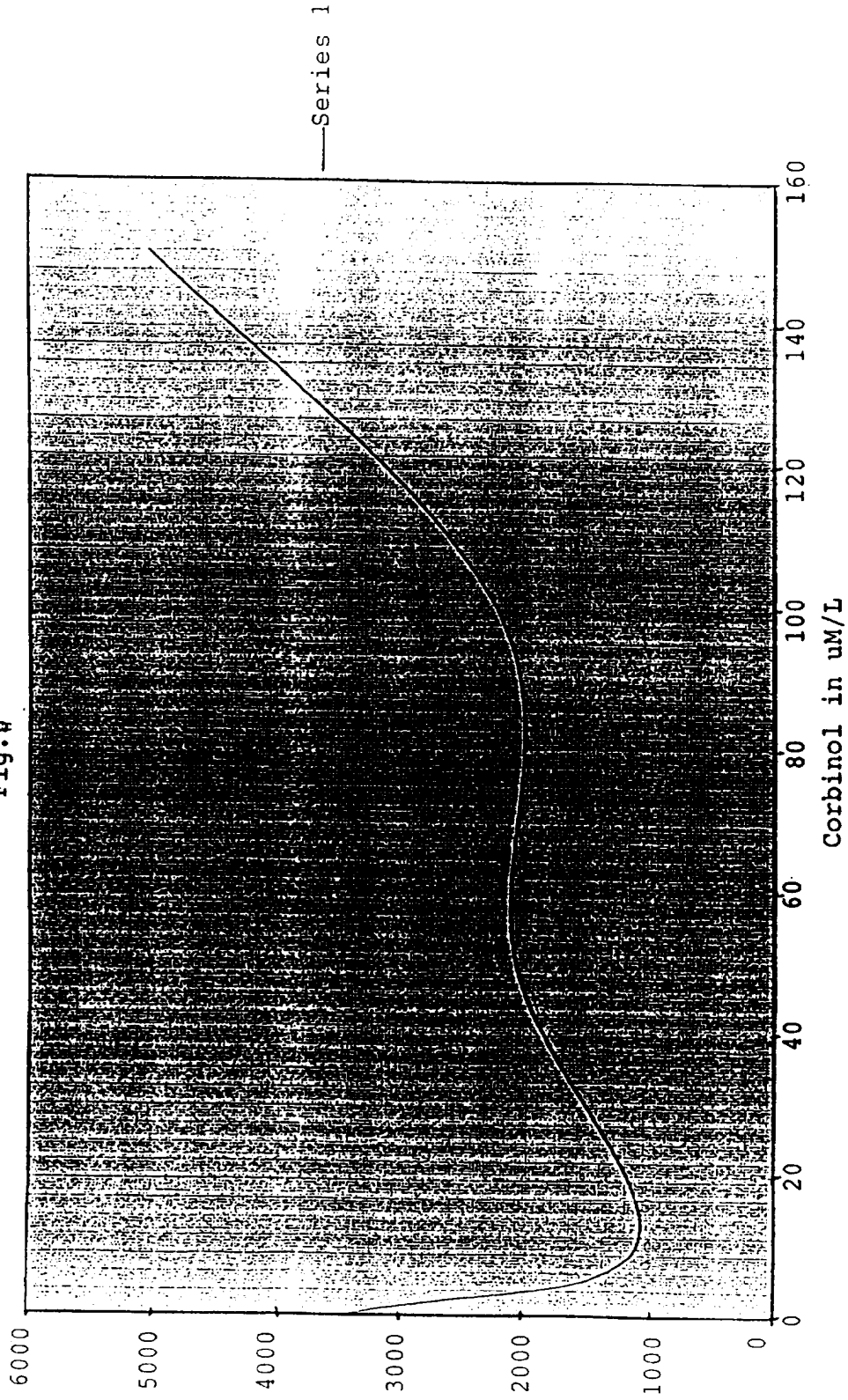



Fig.5

| | INTEGRAL |
|---|---|
| A | 1.00 |
| B | 1.921 |
| C | 0.594 |
| D | 3.989 |
| E | 4.267 |
| F | 2.184 |
| G | 2.278 |
| H | 29.639 |
| I | 0.308 |
| J | 3.142 |
| K | 1.278 |

Influx of Inulin into BHK cells

Fig.6



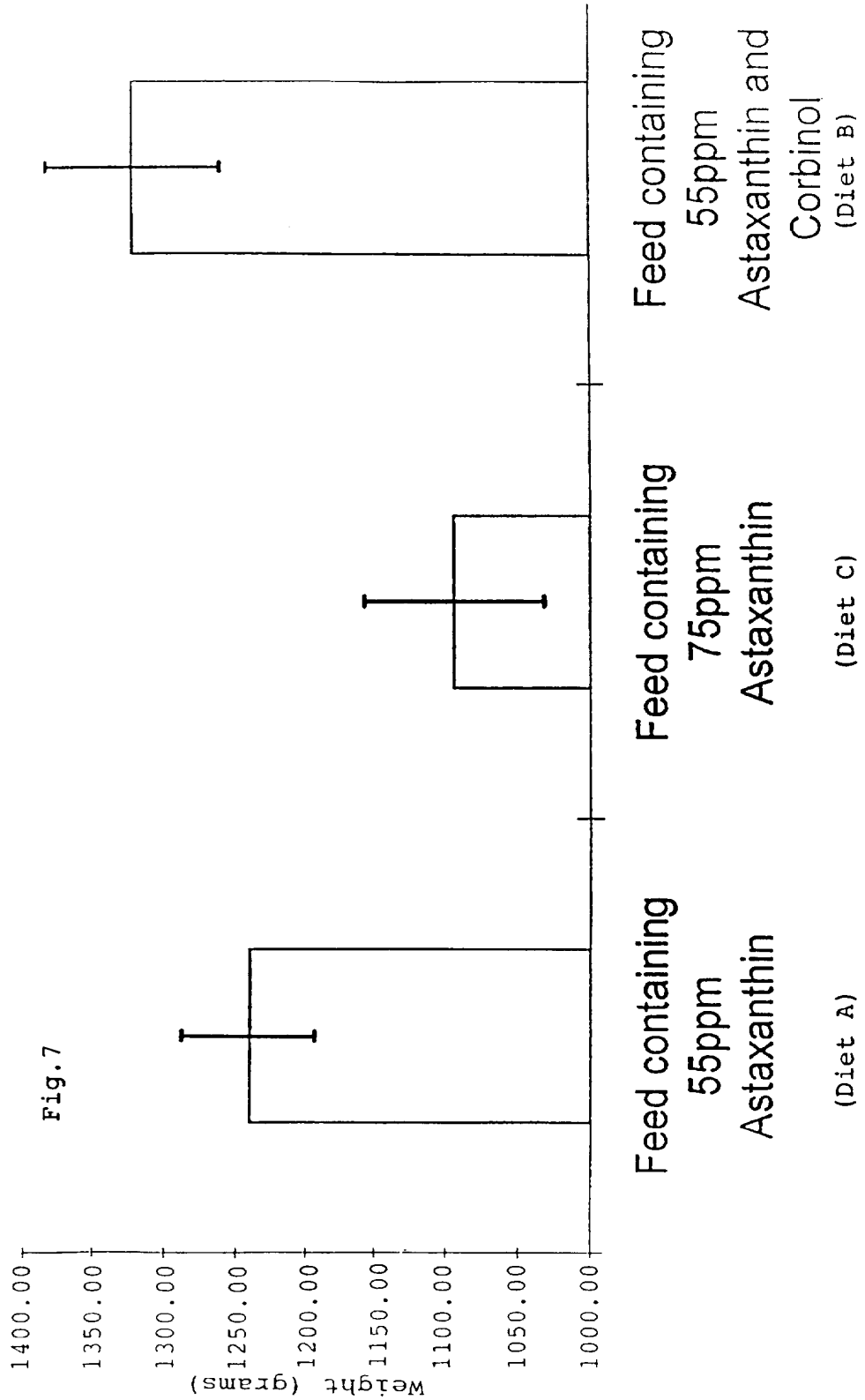

Weight (grams)
1400.00
1350.00
1300.00
1250.00
1200.00
1150.00
1100.00
1050.00
1000.00
Feed containing 55ppm Astaxanthin
(Diet A)
Feed containing 75ppm Astaxanthin
(Diet C)
Feed containing 55ppm Astaxanthin and Corbinol
(Diet B)

Fig.7

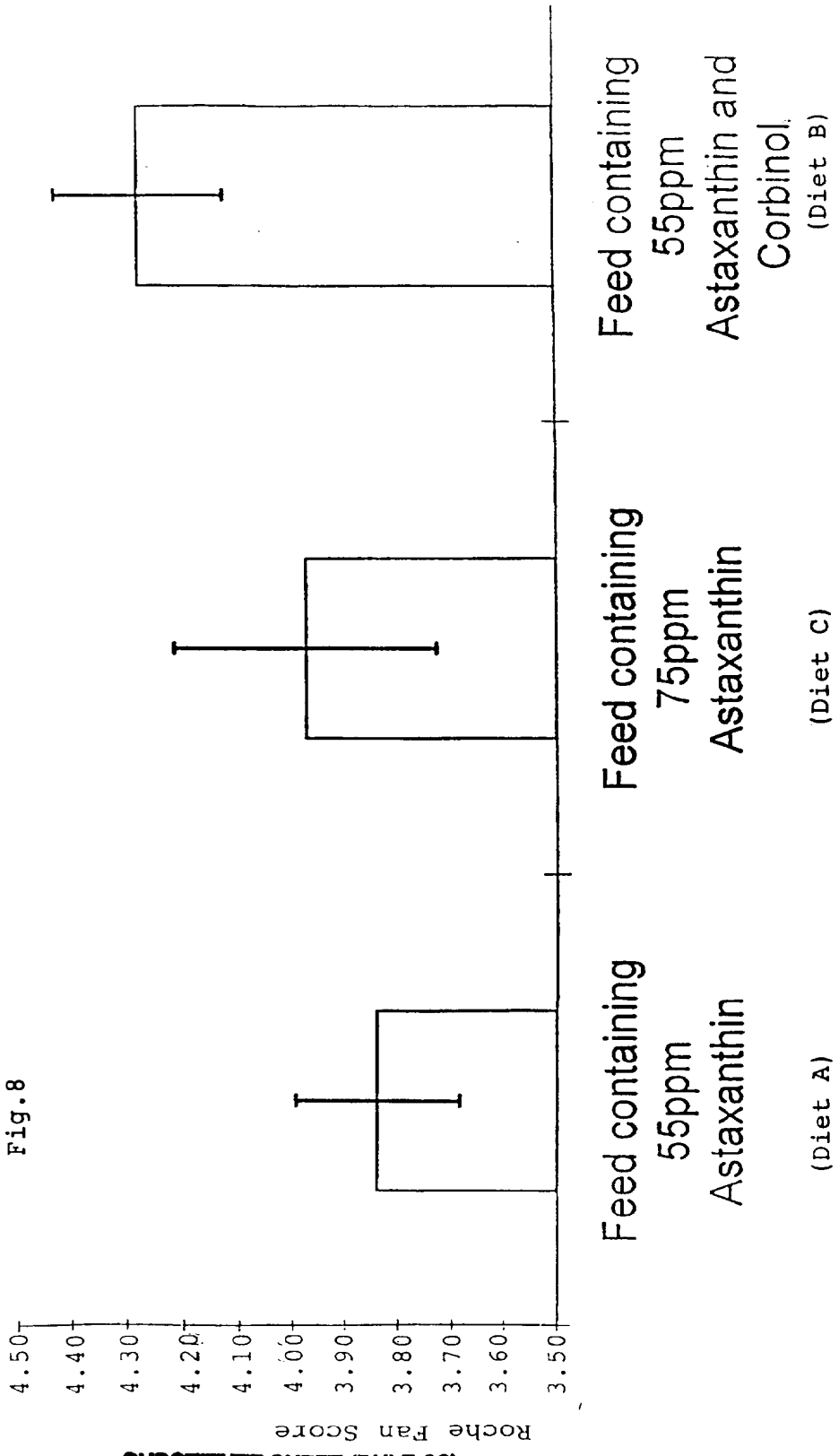

Roche Fan Score
4.50
4.40
4.30
4.20
4.10
4.00
3.90
3.80
3.70
3.60
3.50
Feed containing 55ppm Astaxanthin
(Diet A)
Feed containing 75ppm Astaxanthin
(Diet C)
Feed containing 55ppm Astaxanthin and Corbinol
(Diet B)

Fig.8

# INTERNATIONAL SEARCH REPORT

Inte ›nal Application No
PCT/GB 97/01020

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07F9/38 A61K47/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07F A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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| X | US 2 304 156 A (MAX ENGELMANN ET AL.) 8 December 1942<br>see the whole document | 1 |
| X | DE 25 30 139 A (BENCKISER GMBH JOH A) 20 January 1977<br>see page 2; claim 1 | 8 |
| A | WO 94 22324 A (GARNETT DAVID) 13 October 1994<br>cited in the application<br>see page 2 - page 5 | 1,16 |

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

1

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 10 July 1997 | 31 -07- 1997 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. ( + 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: ( + 31-70) 340-3016 | Rufet, J |

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# INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 97/01020

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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Information on patent family members

Int ional Application No
PCT/GB 97/01020

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